

# Exposure to Aflatoxin B<sub>1</sub> in Animal-Feed Production Plant Workers

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The exposure to aflatoxin B<sub>1</sub> (AFB) in animal-feed processing plants was assessed using binding of AFB to serum albumin. The albumin fraction was digested with pronase, and the digest was purified on a C18 Sepak column and an aflatest affinity column before quantification by ELISA. The level of detectability was 5 pg/mg albumin. The workers served as their own controls, as blood samples were taken upon return from vacation and after 4 weeks of work. A total of 7 of 45 samples were positive for AFB, with an estimated average daily intake of 64 ng AFB/kg body weight. The exposed workers had been disembarking cargos contaminated with AFB or working at places where the dust contained detectable amounts of AFB. The sera from the exposed workers had a significantly higher titer against an aflatoxin B<sub>1</sub>-epitope than a nonexposed Danish control group. The level of exposure could partly explain the increased risk of liver cancer in workers in the animal-feed processing industry.

## Introduction

Aflatoxin B<sub>1</sub> (AFB), a potent liver carcinogen in experimental animals, is produced by *Aspergillus flavus* and *A. paraciticus* under warm and humid conditions. A causative association between the exposure to aflatoxins and human cancer has been established in Dutch workers in an oil mill processing peanut and linseed oil, especially because the incidence of lung cancer was higher than expected (1). A significant excess of primary liver cancer was observed in Swedish workers in grain mills, and it was postulated that exposure to aflatoxins may play a role (2). Similarly, an increased incidence of liver cancer has been reported among male Danish workers in livestock-feed processing companies (3). Exposure to aflatoxins through the respiratory route in these workers was estimated to 170 ng/day based on conservative estimates. Attempt to detect AFB or its metabolites in urine of potentially exposed workers using an enzyme-linked immunosorbent assay (ELISA) was unsuccessful due to the presence of aflatoxinlike antigenic material (4).

Aflatoxin B<sub>1</sub> is metabolized by the mixed-function oxidase system to a number of hydroxylated metabolites and to the 8,9-epoxide. The latter is considered the ultimate carcinogenic form that reacts with cellular DNA and proteins to form covalent adducts. Detection and quantification of these adducts have been suggested as alternative methods to detect human exposure to

aflatoxins (5). Using an ELISA and a monoclonal antibody against AFB, AFB bound to albumin could be detected in blood samples collected in Gambia and Kenya, but not in samples collected in France (6). The sensitivity of the assays were 5.0 pg AFB/mg albumin (5,6). In the present paper we have used a combination of affinity purification of pronase-digested albumin and an ELISA to detect aflatoxin-albumin adducts in workers potentially exposed to AFB.

## Subjects and Methods

### Population

A total of 45 workers at two different livestock-feed processing companies in Esbjerg, Denmark, 15 at company A and 30 at company B, were included in the study. The workers were working either in the factory and silo (29 people) or in the harbor unloading ships with raw material for the production (16 people). They were all males 25-62 years old. None of the workers had been working in other agribusinesses for 3 months before the start of this study.

### Analysis of Blood Samples

Blood samples (5 mL) were collected upon return to work after at least 2 weeks of vacation, and then after 4 weeks of work. Serum was isolated from the blood samples by centrifugation, and albumin was precipitated by addition of saturated ammonium sulfate (final concentration 50%). After removal of globulins by centrifugation, albumin was precipitated from the supernatant by acidification with acetic acid.

Albumin was redissolved in 500  $\mu$ L 0.1 M sodium phosphate buffer, pH 7.4, and was digested with pronase (Fluka, 25 units

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per 5 mg albumin) for 18 hr at 37°C. The concentration of albumin was determined by the Biuret reagent (Sigma kit 540-2) assay. The digest was applied to a prewet C18 Seppak column (Waters, Milford, MA). The column was washed with 5 mL 5% methanol to remove small peptides and amino acids, before the AFB-containing fraction was eluted in 80% methanol (5 mL). This fraction was evaporated to 0.5 mL and was diluted to make 10% methanol (1 mL). The eluate was applied to an Aflatest affinity column (Vicom, MA), and the AFB containing fraction was eluted with 1.5 mL of methanol in phosphate buffer 0.1 M, pH 7.4 (1:1), after rinsing the column with phosphate-buffered saline (PBS; 5 mL).

## ELISA Assay

Polystyrene microtiter plates (Teknunc, Roskilde, Denmark) were coated with AFB-BSA (bovine serum albumin; Sigma) or BAS as previously described (4). On each plate, 100  $\mu$ L of the eluates and five AFB<sub>2</sub> (25; 2.5; 0.25; 0.025; 0.0 ng/mL) standards were added immediately before 100  $\mu$ L of a solution of monoclonal antibody AW1 (diluted 1:1000 with PBS; 7) was applied to compete for binding. The plates were incubated overnight at 4°C, then washed with 0.05% Tween 20 in PBS before incubation with the secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (Dakopatts, Glostrup, Denmark; diluted 1:1000 in 1% BSA in PBS) for 2 hr at room temperature. After washing with 0.05% Tween 20 in PBS and 0.1 M diethylamine buffer (pH 9), 100  $\mu$ L of 4-nitrophenylphosphate substrate (Sigma; 1 mg/mL) was added to each well, and the plates were incubated for 2 hr at room temperature. The absorption was read at 405 nm.

## Results and Discussion

The sensitivity of the analysis using Seppak prepurification and affinity chromatography before quantitative analysis using ELISA was 5 pg AFB/mg albumin, corresponding to 30% inhibition. It was assumed that the antigenicity of AFB-amino acids was similar to AFB<sub>2</sub> using the AW1 antibody, as an epitope mapping indicates that modification at the 7,8- position only had minor influence on the antibody recognition (4). Five milligrams of serum albumin per incubation could be used without influencing the quantification.

One of the advantages of using serum proteins compared to lymphocyte DNA is that the measured level represents the accumulated dose during the half-life of the blood proteins, as no repair pathways are known, whereas the half-life of aflatoxin-DNA adducts in experimental animals is less than 24 hr. A good correlation between the binding of AFB to blood proteins and liver DNA has been reported in experimental animals, suggesting that binding to a serum protein is a good monitor for the binding to target cell DNA even at low doses of AFB. It has been estimated that approximately 5% of the administered dose binds to albumin (6).

Blood samples were collected from workers with different suspected exposure to AFB-contaminated material as based on job descriptions. Two of the 45 serum samples collected from the workers upon return from vacations indicated recent exposure to AFB, 50 and 54 pg/mg albumin, whereas all the other samples were below the detection level of 5 pg/mg albumin. After 4 weeks

**Table 1. Individuals exposed to aflatoxin B<sub>1</sub> (AFB) measured as AFB-albumin adducts.**

Worker	Company/ work area	Working hours in contaminated areas*	pg AFB/mg albumin	
			1st Blood sample	2nd Blood sample
1	B, silo	L	ND	58
2	B, silo	S/L	ND	64
3	B, factory	S	54	44
4	B, factory	L	50	ND
5	B, factory	S	ND	50
6	B, dock, team 2	S	ND	88
7	B, dock, team 2	S	ND	100
8	B, dock, team 2	S	ND	50
9	B, dock, team 2	S	ND	ND

ND, not detectable; less than 5 pg/mg.

\*L, less than 25% of working hours; S, more than 50% of working hours.

of work, 7 out of 45 workers had detectable levels of AFB, suggesting an occupational exposure to AFB (Table 1).

The mean level of exposure for a worker, only including the individuals with detectable levels, was 64 pg AFB/kg/day, assuming that 5% of the dose bound to albumin and that the exposure was equally distributed over the 30 working days between the two blood samples. This estimated level was higher than the level of exposure in an area with intermediate liver cancer rate, where the exposure had been estimated to be 16.5 ng/kg (8).

Three out of the five workers with the highest antigenicity were working on discharging a cargo with an AFB level of 26  $\mu$ g/kg. One of the workers with a detectable level of aflatoxin-albumin adduct but with suspected low exposure based on job description had a supervisory function at the grill, where batches of raw material from different silos were dumped for use in the production. Dust samples collected at this site had an AFB content of 8  $\mu$ g/kg dust.

Assuming that the measured exposure is representative for the AFB exposure through an extended period of many years for the workers at the animal-feed manufacturing companies in Denmark, 85 liver cancer cases per 1 million exposed individual per year would be expected, using the linear regression data from correlation studies on AFB exposure and liver cancer incidences (9). Among the 2000 members of the Trade Unions representing the workers, only 1.7 new cases of liver cancer should be expected based on the monitoring data compared to the observed value of 3 cases (3).

Exposure to carcinogens normally takes place 10–30 years before diagnosis of cancer. The quality of the raw material, the number of cargoes contaminated with aflatoxins, and the working environment have changed considerably in the last 10 years, so that the measurements made today are not representative for the period in which the liver cancer patients were exposed. As only a few of the Trade Unions members are currently exposed, a lower number than 1.7 cases/10 year is to be expected.

It has previously been reported that people living in Kenya with a suspected high exposure to AFB had a high titer of an antibody against an AFB epitope (10). The antibody titer against the AFB epitope was significantly higher in the people working in an environment with potential exposure to AFB than in nonoccupationally exposed individuals (Table 2.) The high antibody titer in occupationally exposed individuals may be due to concomitant exposure to endotoxins that act as an adjuvant.

Table 2. Titer of antibody recognizing an aflatoxin B<sub>1</sub> epitope.

Titer	This study	Control Denmark <sup>a</sup>	Kenya <sup>a</sup>
< 5000	24*	17	41
> 5000	18*	1	19
Total	42*	18	60

<sup>a</sup>Data from Autrup and Seremet (10).

\*Significantly different from control Denmark ( $p < 0.005$ ) and Kenya ( $p < 0.032$ ) using Fisher's exact test.

Antibodies against a carcinogen epitope have been shown to decrease the uptake of the carcinogen after oral administration (11), inhibit carcinogen-DNA binding (12), and inhibit tumorigenicity (13). Dietary exposure to aflatoxins alters the immune function (14), but the biological consequence of antibodies against AFB remains unknown.

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